REMARKS

Reconsideration is respectfully requested. Claims 4, 7, 10, 13, 17, and 20-24 are pending. Claims 4 and 7 have been amended. Claims 1-3, 5, 6, 8, 9, 11, 12, 14-16, 18, and 19 have been canceled. Claims 20-23 have been withdrawn. No new matters have been added due to the amendments. Amendment to and cancellation of the claims does not affect inventorship.

Applicants have not dedicated or abandoned any unclaimed subject matter and moreover have not acquiesced to any rejections made by the Patent Office. Applicants reserve the right to pursue prosecution of any presently excluded claim embodiments in future continuation and/or divisional applications.

As a preliminary matter, Applicants thanks the Examiner for acknowledging that claim 24 is allowed. Applicants also amended claim 4 to depend from claim 24, thus it should be allowed as well.

In the Specification

The specification is objected for failure to comply with the requirement of 37 CFR 1.821(a)1(1) and (a)(2) with regard to sequence identifier in the specification, specifically, the description related to Figures 3 to 5. The specification has been amended to include sequence identifiers. As such, the application complies with the requirement of 37 CFR 1.821(a)1(1) and (a)(2) and Applicants respectfully request the rejection be withdrawn.

Claim Objection

Claim 5 stands objected as being a substantial duplicate of claim 24. Claim 5 has been cancelled, rendering the objection moot.

Claims Rejections - 35 U.S.C. § 112, First Paragraph

Claims 1, 4, 7, 10, 11, and 13 stand rejected under 35 U.S.C. § 112, first paragraph as failing to comply with the written description and enablement requirements. Applicants respectfully traverse.

Claims 1 and 11 have been cancelled, rendering the rejections moot. Claim 4 has been amended to depend from allowed claim 24, and as such is also allowable.

With regard to claim 7 (and claims depend therefrom), Applicants respectfully direct the Examiner to M.P.E.P. 2163, which states:

> The written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice.
>
> The Examiner states that:
>
> Applicants ... were not in possession of the genus claims such as ... method of obtaining a specific crystal grew under any crystallization conditions. The disclosure of a well-known method for screening crystallization conditions is no indication that applicants were in possession of the entire genus of crystals or method of crystallization.

Applicants respectfully disagree. Claim 7 has been amended to recite "a protein crystal is formed that has a crystal lattice in a $P2_12_12_1$ space group and unit cell dimensions, +/- 5%, of a=68.7 b=79.6 c=150.2, $\alpha=\beta=\gamma=90$." Thus, it claims a genus method to grow this specific crystal of a protein. As stated in M.P.E.P. 2163:

> A "representative number of species" means that the species which are adequately described are representative of the entire genus.
>
> ....
>
> What constitutes a "representative number" is an inverse function of the skill and knowledge in the art. Satisfactory disclosure of a "representative number" depends on whether one of skill in the art would recognize that the applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus in view of the species disclosed.... Description of a representative number of species does not require the description to be of such specificity that it would provide individual support for each species that the genus embraces.

Moreover, as the Examiner can appreciate, at the beginning of this new millennium, the time the instant application was filed, "the skill and knowledge in the art" required for growing crystal had come a long way from the early age of crystallography, or even a decade before then. Kits for large scale screening were widely commercially available, and methods and equipments for robotic large scale screening have been developed by entities such as the assignee of the instant application, Syrrx Inc. Due to these developments, large scale screening with minimal amount of protein for conditions to grow crystals became routine, particularly in the industry. For example, Walter T.S., J. Appl. Cryst. 36:308-314, (2003) (Attached as Exhibit A) described a protocol that "allows crystallization experiments to be set up at a throughput of up to about 4000 drops in a working day using commercially available equipment with only minor modifications." At page 314, right column. (Emphasis added).

Applicants further direct Examiner to pages 41-46 of "Crystallography Made Crystal Clear, G. Rhodes, 3rd Ed. (2006) ("*Rhodes*") which is attached as Exhibit B. *Rhodes* discloses a sample scheme for finding optimum crystallization conditions and that "multiple batches of crystals can be grow conveniently by the hanging-drop or other methods." See Pages 41-42 and Figure 3.8. It also discloses that the crystallization condition can be plotted on a four-dimensional surface, where the optimal conditions are represented by the peak on the surface. See Figure 3.9.

As such, as the time the instant application was filed, "the skill and knowledge in the art" had grown into a stage that it was not necessary to disclose each and every condition, foreseeable or unforeseeable, to meet the ""representative number" requirement. Description of a representative number of conditions to grow the claimed specific crystal does not require the description of such specificity each growing condition. The wide range of crystallization conditions disclosed by the present invention is sufficient to provide guidance to further explore for new conditions to grow the claimed crystal.

Applicants further submit that a sufficient "representative number" of conditions - the conditions to grow the specific crystal as recited in claim 7- have been provided by the instant application. The specification discloses that the Applicants have undertaken "systematic broad screening crystallization trials on an MvaS complex using the sitting drop techniques" to obtain conditions that appear to produce precipitate and/or crystals. These conditions are used for fine screening for optimal conditions. See paragraphs [0090] and [00181]. These experiments led to a thorough understanding of how crystallization conditions affect MvaS crystallization, and a series of crystallization conditions were identified that maybe used to for crystals comprising MvaS. These conditions are summarized in Table 5. The specification further discloses in details one of the crystals obtained under one of the disclosed conditions. *See* Example 2. In addition, the specification disclosed that these crystals could also be obtained with different type of PEG and at different temperatures. *See* Example 2. Therefore, the Applicants have disclosed a wild range of conditions that can be used to grow the claimed specific crystal.

Thus, at the time of the filing, Applicants not only were in possession of the claimed genus method, but also provided sufficient guidance to enable a skilled artisan to practice the claimed method without undue experimentation. As such, the rejection is improper and should be withdrawn.

CONCLUSION

Applicants respectfully submit that the claims are now in condition for allowance and early notification to that effect is respectfully requested. If the Examiner feels there are further unresolved issues, the Examiner is respectfully requested to phone the undersigned at (415) 442-1000.

Respectfully submitted,

Dated: 05/14/2007

By: _____

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A procedure for setting up high-throughput nanolitre crystallization experiments. I. Protocol design and validation

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A protocol for setting up nanolitre sitting-drop vapour-diffusion experiments is reported. The procedure uses standard crystallization screening kits and 96-well crystallization plates. Reservoir solutions are transferred from 96-deep-well blocks to crystallization plates in a single step with a Robbins-Hydra pipettor. Nanolitre droplets of protein as well as reservoir solution are dispensed by a Cartesian pipetting instrument. Experiments have been carried out to characterize the performance of this instrument. Adaptations to the Cartesian, which include an anti-evaporation cover plate, are described and tested. The protocol was designed for a high-throughput facility, but can be used in any standard crystallography laboratory.

1. Introduction

A major rate-limiting step in protein crystallography is the production of diffraction-quality crystals. The initial identification of crystallization conditions is typically achieved by screening a range of parameters such as protein concentration, pH and type of buffer, type and concentration of precipitants, ions and cofactors, and temperature. This screening stage often uses substantially more protein than is subsequently needed to hone conditions to produce the crystals used for diffraction analysis. Screening biological macromolecules to identify crystallization conditions for X-ray diffraction analysis is thus labour-intensive and both time- and protein-consuming, dictating the minimum amount of protein required for crystallographic analysis. In order to reduce the human labour required and to improve reproducibility, automated screening systems have been investigated by several groups (Chayen *et al.*, 1990; Snook *et al.*, 2000; Villaseñor *et al.*, 2002). The use of automation in conjunction with ink-jet technologies (Tisone, 1998; Rose, 1999) also opens up the possibility of using smaller nanolitre-scale screening experiments. Robotic systems for the dispensing of very small drops for crystallization experiments are currently being developed and used by high-throughput structure-determination-focused companies, groups and consortia (Kuhn *et al.*, 2002; Sulzenbacher *et al.*, 2002). Such systems will lead to substantial savings in protein used and a potential increase in the number of conditions that can be screened. Although smaller crystals might result from such experiments, they may be of better quality or be of different forms (Santarsiero *et al.*, 2002). Indeed some proteins may only crystallize in smaller volume experiments (Bodenstaff *et al.*, 2002). Should crystals arising from such techniques be too small for X-ray crystallographic analysis, they may still be used for seeding. However, we note that various developments at synchrotron sources and the use of cryo-cooled crystals allow excellent quality diffraction data to be collected from crystals with volumes at least an order of magnitude less than would have been necessary a decade ago.

Here we describe adaptations to, and the use of, a Cartesian MicroSys dispensing instrument for setting up 100 nl-scale vapour-diffusion sitting-drop crystallization experiments in 96-well plates. Used in conjunction with a Robbins Hydra microdispenser to set up the reservoir solutions, the Cartesian MicroSys provides an affordable and accurate platform with throughput appropriate to an active structural biology laboratory.

2. Methods and materials

2.1. Crystallization plates, screening kits and reagents

Crystallization experiments were carried out in 96-well crystallization plates from Greiner (reference number 609.101, Greiner Bio-One Ltd, Stonehouse, UK) using the vapour-diffusion method. With this plate it is possible to carry out 96 separate crystallization experiments on the footprint of a standard microtitre plate. Each well contains space for over 100 µl of reservoir solution and three small individual platforms for the protein droplet. The plate used in the present experiments had square protein wells and flat bottom platforms. This type of plate is compatible with automated imaging systems.

Crystallization plates were sealed with transparent self-adhesive plastic foils as supplied by Greiner (Viewseal). These

foils contain an encapsulated sealant, which is released only in those areas where the foil is pressed against a support. The amount of sealant vapour, which may interfere with the crystallization process, is thereby significantly reduced. The seals are not permanent, as compared with heat seals, and foils can be taken off and re-applied.

Standard crystallization screening kits, purchased from Hampton Research (CA, USA) and Emerald BioStructures (deCODE Genetics, WA, USA), were assembled into deep-well blocks (Greiner, 2 ml Masterblock-PP) of 96 reagents. This 96-well format can be processed by the Hydra and Cartesian pipetting instruments.

Five standard blocks were formatted to contain the following kits.

Block 1: Hampton Research Crystal Screen, Hampton Research Crystal Screen II.

Block 2: Emerald BioStructures Wizard I, Emerald BioStructures Wizard II.

Block 3: Hampton Research PEG/Ion Screen, Hampton Research Grid Screen PEG 6000, Hampton Research Grid Screen Ammonium Sulfate.

Block 4: Hampton Research Natrix Screen, Hampton Research Crystal Screen Cryo.

Block 5: Hampton Research Grid Screen PEG/LiCl, Hampton Research Grid Screen NaCl, Hampton Research Grid Screen MPD, Hampton Research Quik Screen.

Deep-well blocks were sealed with a metal sealing foil (Adhesive PCR Sealing Foil, Product Code AB-0626, Abgene, Epsom, UK). This type of foil was also applied to the black microtitre plates used for the fluorescence intensity measurements. Other reagents used were: AnalaR-grade 2-propanol (BDH, Poole, UK) for viscosity experiments; MilliQ-quality water for all buffers and the Cartesian system fluid; PEG8000 in a 50%(*w*/*v*) stock solution; Fluorescein (Product Number F7505, Sigma, Poole, UK).

2.2. Pipetting hardware

2.2.1. Reservoir dispensing. The instrument used to pipette the reservoir solutions was a Hydra-96 microdispenser (Apogent Discoveries, Wilmslow, UK). The Hydra has 96 syringes (of 290 µl each) fitted with Duraflex needles and dispenses 96 solutions simultaneously. It was necessary to control the instrument from a computer *via* an RS232 port, so that all operations, such as syringe filling times and table movements could be selected individually. By applying the appropriate filling time it is possible to pipette even highly viscous solutions accurately. An adaptor block was installed so that the syringes were positioned centrally above the Greiner plate reservoir area and did not collide with the side walls of the plate.

2.2.2. Nanolitre droplet dispensing. A Cartesian Technologies dispensing instrument (Microsys MIC4000, available from Genomic Solutions, Huntingdon, UK) was used to set up the nanolitre droplets. The instrument has a head of eight ceramic tips, which can dispense nanolitre droplets individually as well as simultaneously. The table (or bed) has space for three 96-well plates and also contains the wash and drying station for the tips. In the present arrangement only one of the three possible spaces is used (position 2). The table can be moved in the *x* direction and the head in the *y* and *z* directions (see Fig. 1). This makes it possible to pipette drops to any position on the plate. All processes are controlled *via* a computer using the Cartesian *AxSys* software.

The Cartesian system is based on ink-jet technology using fast solenoid valves (Rose, 1999). Syringes create a steady-state pressure within the system fluid and by opening and closing the valves small droplets can be ejected from the tips. Before pipetting the system has to go through several steps, including pre-pressurization and pre-dispensing to reach a steady-state pressure in the system fluid. This steady-state pressure is essential to obtain consistent and accurate drop sizes. For a given pressure the desired drop volume is then obtained by choosing an appropriate valve opening time. The valve opening time was 1000 µs for 100 nl and 583 µs for 50 nl drops. Two different modes of dispensing are used for crystallization experiments: line-dispense for the protein and single-dispense for the precipitant droplets. Both modes are non-contact pipetting methods.

In line-dispense mode, drops are pipetted while continuously moving the table ('on-the-fly'). This method of dispensing is fast and accurate because the opening of the valve is synchronized with the table movement. For crystallization experiments, an aliquot of protein solution is aspirated by one of the eight tips (in our case tip No. 7) and this tip then dispenses small droplets of protein to all 96 crystallization platforms on-the-fly.

In single-dispense mode, the table is first moved to the selected position before the drop is pipetted. The dispense head can also be moved up or down between drops. This mode is used to aspirate reservoir solutions from the Greiner wells and then pipette small droplets onto the corresponding platforms. As the spacing between the tips conforms to the stan-

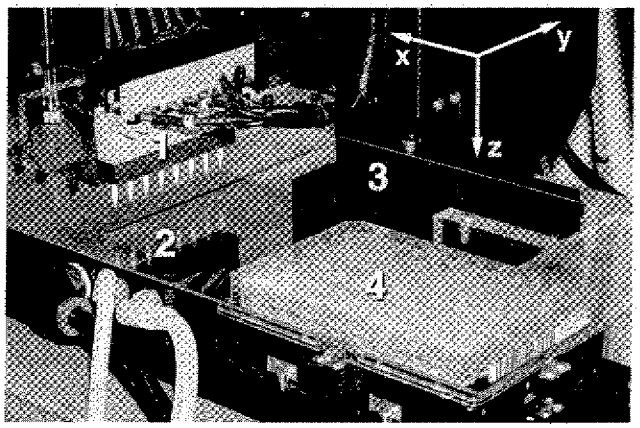

Figure 1
Close-up of the Cartesian pipetting instrument, showing the dispense head with the row of eight ceramic tips (1), the wash and drying station (2), and the anti-evaporation cover (3), with a 96-well crystallization plate (4) in position on the table. During the pipetting operation the table moves in the *x* direction, while the dispense head moves in the *y* and *z* directions, as indicated.

dard 96-well plates, columns of eight reservoir solutions can be pipetted simultaneously. After each pipetting step the tips need to be rinsed and cleaned at the wash station before the next column can be pipetted.

2.2.3. Adaptation to equipment. To limit evaporation, a humidity chamber is available from the supplier. This chamber did not seem appropriate for our application where individual wells may contain different reservoirs, each with its own specific vapour pressure and humidity. A device in the form of a plastic sheet was built and fitted to the instrument such that the top of the Greiner plate is covered (Fig. 1). The cover is made of polycarbonate, a strong and virtually unbreakable plastic with excellent optical properties, making it easy to inspect the droplets while the Greiner plate is under the cover. A narrow slot, positioned precisely underneath the tips, allows all pipetting operations to be carried out without touching the cover. Because the cover is attached to the body of the Cartesian instrument it remains stationary, whereas the crystallization plates can slide underneath it when the table is moved.

With this adaptation in place, individual wells remain covered for most of the time. They are only exposed when the instrument pipettes solutions into a column of wells and only during the actual time of pipetting. While dispensing the protein droplets individual wells will only be open for a very short time, because line-pipetting is fast and pipetting is carried out in the x direction, whereas the slot of the cover is in the y direction. For the reservoir pipetting the cover-open time may be more significant because the aspirate, pre-dispense and dispense process for each well is relatively slow (some 45 s).

A spacer block was installed on the stage of the Cartesian to bring the top of the Greiner plate to a slightly higher level than the wash station. The cover limits the down-movement (z-axis) of the tips, which meant that a slight modification to the wash station had to be made to make sure that the tips are fully immersed in the washing liquid. An additional water trap had to be installed in the vacuum line, used to dry the tips, to protect the vacuum pump. In the absence of a cover, water from the wash station would not normally carry over to the vacuum line, but with the cover this can easily happen.

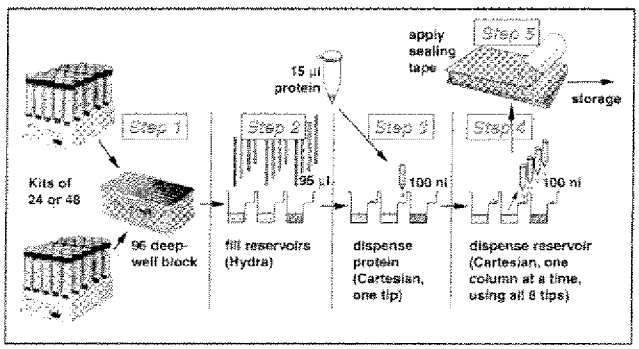

Figure 2
Protocol for setting up crystallizations.

2.3. Measurement of drop volumes and drop evaporation

Physical measurements were carried out to determine the overall volume of aspiration and dispensing. For aspiration the sample tube was weighed before and after aspiration. To determine the overall dispense volume the sample was line-dispensed repeatedly on to a flat plate and the plate was weighed before and after dispensing. Drops were pipetted onto a thin layer of low-density mineral oil (Sigma, Poole, UK) to prevent evaporation.

Volumes of individual drops were determined by adding a fluorescent dye (fluorescein) to the sample and measuring the fluorescence intensity of the dispensed drops. Because the intensity of fluorescein is pH-dependent, the dye was kept in a 20 m*M* Tris-HCl, pH 8.0, buffer at all times. Nanolitre drops were pipetted into black 96-well microtitre plates (Nunc, Roskilde, Denmark), which contained 95 µl of buffer in each well. Plates were sealed with metal-sealing foil until fluorescence readings could be taken. The fluorescein concentration of the aspirated solutions was 0.1 mg ml^{-1}, from a stock solution at 1 mg ml^{-1}.

A SpectraFluor Plus fluorescence spectrophotometer (Tecan, Reading, UK) was used (three flashes, 40 µs integration time, 60× gain) to obtain relative fluorescence intensity readings. These were converted to volumes by use of a calibration curve, which was obtained by measuring the fluorescein of hand-pipetted solutions under the same conditions at the same time.

A Zeiss stereomicroscope in reflected-light mode was used to monitor drop sizes over time. A cold light source was used to avoid heat transfer to the drops, which could influence the evaporation rate. Drop diameters were measured with a graticule eye-piece and recorded at 30 s intervals. During the measurement the plate remained stationary under the microscope.

3. Results and discussion

3.1. Protocol for setting up crystallization screens

The protocol for setting up nanolitre crystallization experiments is shown in Fig. 2.

3.1.1. Reformatting screening blocks. The first step involves reformatting and pipetting solutions from standard screening kits to 96-deep-well blocks. At present this is carried out by hand. The blocks are then sealed and stored at room temperature until further use. Five blocks of 96 different reagents are now routinely used (contents are as described in §2). Blocks containing other screening kits or customized solutions can be made up at this stage.

3.1.2. Dispensing the reservoir. In the second step, the screening solutions are transferred from the deep-well blocks to the Greiner plate with a Robbins-Hydra instrument. By aspirating 285 µl of solution with each syringe from the deep-well blocks it is possible to fill the reservoirs of three Greiner plates (at 95 µl each). This operation is quick because the Hydra has 96 syringes which are operated in parallel. The time required is essentially determined by the syringe filling time,

which can be up to 2 min for viscous solutions. Greiner plates are then transferred directly to the Cartesian instrument or sealed with metal foil (as above) and stored. Should a uniform dilution of reservoir solution with water or buffer be required, this can be also carried out with the Hydra.

3.1.3. Dispensing the protein solution. Steps 3 and 4 involve nanolitre dispensing of protein and reservoir solutions and are performed with the Cartesian Microsys. The Greiner plate containing reservoir solutions is placed on the Cartesian table and moved by the table to a position underneath the plastic cover sheet. In all subsequent operations of the Cartesian the plate is kept under this cover to prevent drop and reservoir evaporation.

In step 3 the protein solution is aspirated by tip No. 7 and dispensed 'on-the-fly' to all 96 Greiner platforms. Only the middle platform of the Greiner plate is used for this; the other two positions remain empty and are available for other experiments, such as mixing additives/detergents with the reservoir solution or performing soaking/cryo-experiments. With this procedure any one plate contains only one particular protein, which eliminates mistakes with sample identity. At present the routine operation is carried out with droplets of 100 nl, but smaller or larger drops can be dispensed, if required. To pipette all 96 drops of 100 nl for one plate requires some 15 µl of protein solution and takes approximately 30 s.

3.1.4. Dispensing precipitant. In step 4, precipitant solutions are aspirated from the reservoir and then dispensed on top of the protein droplet. This operation is carried out in single-dispense mode with all eight heads pipetting in parallel. After each dispensing step the tips need to be rinsed and cleaned at the wash station before the next column of eight can be pipetted. This process has to be repeated 12 times to complete one plate and is therefore the rate-limiting step of the whole procedure, requiring about 10 min. At present, reservoir droplets of 100 nl are routinely used. After pipetting the reservoir solutions, plates are sealed with transparent foil (step 5) and moved to the plate storage area.

3.2. Practical experience using this protocol

At present, step 1 (Fig. 2) is carried out by manual pipetting using standard commercial kits, although some kits are already available as 96-deep-well blocks. Starting with the standard kits is more economical and gives more flexibility. Pipetting with the Hydra (step 2) has been found to be fast and reliable. By applying the appropriate filling time it is possible to pipette even highly viscous solutions accurately, but it is essential that all operations can be controlled individually from a computer. Care has to be taken with the up-and-down movement of the table, so that the plate does not collide with the syringe needles. The Duraflex needles have a high degree of flexibility, but if they are bent too much they will retain a permanent kink and need to be replaced.

The Cartesian instrument is now in routine use by many researchers in our structural biology laboratory and generally performs well (Brown *et al.*, 2003). The system fluid should be properly degassed to avoid air bubbles and filtered to prevent fibres becoming caught in the tips. The ceramic tips can become blocked relatively easily, especially when dispensing high salt or polyethyleneglycol (PEG) solutions. Before starting an experiment the dispense head should be checked so that all tips are accurately aligned and dispense properly. A test dispense run of water drops can be performed to check that all drops are placed into the centre of the Greiner platform. Small adjustments to the position of the plate can be made at this stage should the drops indicate that the plate is out of alignment.

In the original protocol, reservoir droplets were dispensed before the protein. It was found, however, that the protein droplets are placed more accurately into the centre of the Greiner platform than the reservoir solutions; hence our present practice of dispensing protein first and reservoir second. The results of nanolitre crystallization experiments carried out with this setup are described in the accompanying paper (Brown *et al.*, 2003).

3.3. Compatibility with automated high-throughput storage and imaging systems

The present setup has been designed to be part of an automated high-throughput system being established at the Oxford Protein Production Facility. All crystallization plates are barcoded. The barcode is read and recorded each time the plate is transferred to a new instrument, so that each step can be traced and documented. Data from the experimental processes are collected and organized by a laboratory information management system (LIMS, Nautilus, Thermo LabSystems, Altrincham, UK), which is currently being set up.

After step 5 (Fig. 2), crystallization plates are loaded into a robotic storage vault (The Automation Partnership, Royston, UK) and images of the droplets recorded at regular intervals with an image acquisition system (OASIS 1700, Veeco, Cambridge, UK). Once the plates enter the vault all subsequent operations will be fully automated and should not require any manual intervention. The imaging system has been set up to take one image of each middle platform of a Greiner plate. To obtain clear images suitable for automated image analysis, the plate must be optically completely transparent at this position. It seems that only plates with flat platforms have the required optical properties. Image-recognition software has been developed (Spraggon *et al.*, 2002; Wilson, 2002) and is currently being tested.

3.4. Dispensed drop volumes

Tests were carried out to check the accuracy and reproducibility of the Cartesian instrument when dispensing small droplets. All operations were performed with the recommended pre-dispense and valve-opening time parameters. Drop volumes were determined by measuring the fluorescence of the added dye fluorescein.

Fig. 3 shows a typical graph of the 96 drop volumes as dispensed with one tip (tip No. 7) in line-dispense mode, the mode used to pipette the protein solution. Some variation in

the individual drop volume was detected but the average volume was close to, although generally lower than, the set volume of 100 nl. This may indicate that the valve opening should be set slightly longer than the recommended time of 1000 µs. The first couple of drops were sometimes smaller than average, possibly because the steady-state pressure had not been fully attained. Experiments performed to determine the total aspirated and dispensed volumes by weight were essentially in agreement with the fluorescence measurements.

The drop volumes pipetted in single-dispense mode by all eight tips are shown in Fig. 4. There was little variation between tips and drop volumes were in general very close to 100 nl. Standard deviations were considerably smaller than in line-dispense mode (see Fig. 3). Since crystallization screens contain a wide range of solutions of different viscosities, dispensed volumes were measured for solutions containing polyethyleneglycol 8000 and 2-propanol (Fig. 5). With PEG8000 the drop volume decreased slightly with increasing PEG concentration. At concentrations of 30 to 35% drops were occasionally not dispensed and at 40% pipetting was no longer reliable. With 2-propanol a more pronounced drop in dispensed volume was observed although this may be due in part to a quenching reaction between 2-propanol and fluorescein.

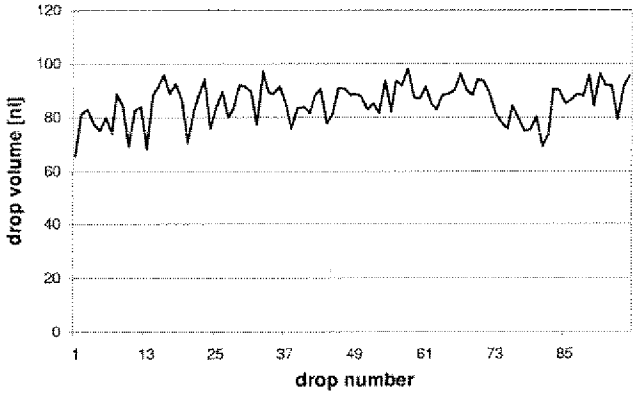

Figure 3
Dispensed drop volumes as a function of drop number. The standard protocol for line-dispensing the protein solution 'on-the-fly' was used. The drop volume was set at 100 nl. The fluorescent dye was present in the aspirated liquid as well as in the system fluid. Volumes shown are individual measurements as pipetted by tip No. 7 in a single process. The average drop volume was 85.4 nl, with standard deviation 7.1 nl.

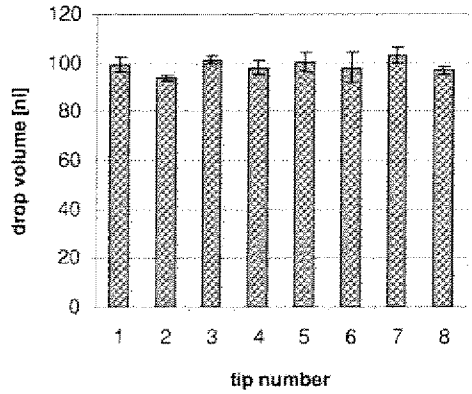

Figure 4
Variation of drop volume for the eight tips using the standard protocol for reservoir dispensing (single-dispense mode and 100 nl drops). The fluorescent dye was present in the reservoir solution. Each value is the average (and its standard deviation) of 12 measurements.

3.5. Well-to-well cross-contamination

As the Cartesian instrument uses a non-contact dispensing method, well-to-well cross-contamination is limited to those cases where solutions are aspirated. Any well-to-well cross contamination of protein can be excluded because each plate contains only one type of protein. There should also be no plate-to-plate carry over since the tips are washed thoroughly before a new protein is used. If cross-contamination occurs it would be when the reservoir solutions are pipetted. Experiments to measure any cross-contamination were carried out as shown in Fig. 6. The carry over of reservoir solutions from one well to subsequent wells was found to be minimal for buffer solution, but significant for 30% PEG8000 solution. This

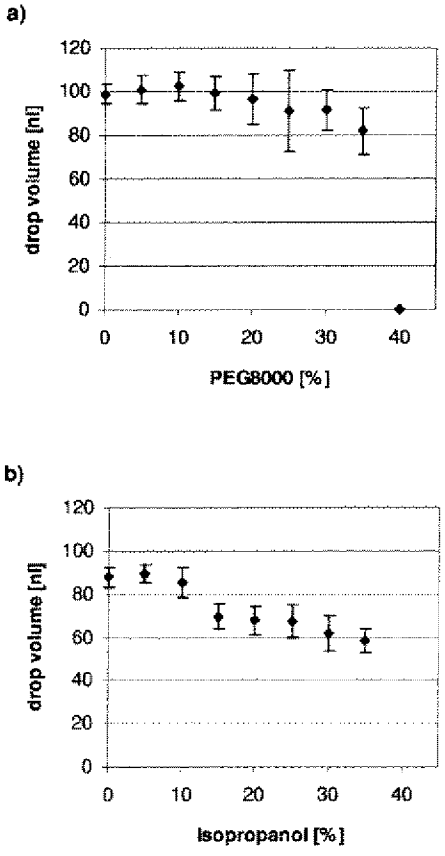

Figure 5
Dispensed volumes for solutions containing (*a*) PEG8000 and (*b*) 2-propanol. The points shown are the average (and its standard deviation) of all 96 drops (using all 8 tips) for one plate. Other experimental details are as described for Fig. 4. With solutions containing 40% PEG8000, drops were not dispensed.

indicates that the present tip-washing procedure is reasonably effective.

3.6. Mixing of protein with system fluid

In the Cartesian instrument, drops are dispensed by applying pressure on to the sample solution *via* the system fluid. In the standard mode of operation the system fluid, usually water, is therefore in contact with the sample and can mix with the sample solution. This mixing is not a problem when dispensing the reservoir solutions, because reservoir solutions can be aspirated in excess and for each reservoir only one drop needs to be pipetted. For the protein solution, however, it is important not to waste hard-won material and to aspirate only the minimum required to dispense 96 drops.

To determine the amount of mixing when dispensing the protein, dye was added to the aspirated solution and the drop fluorescence was measured as a function of drop number. It can be seen from Fig. 7 that the fluorescence intensity decreases substantially over the first 96 drops, indicating a considerable amount of mixing of dye solution with system fluid. Equivalent results of mixing were obtained when the fluorescent dye was present in the system fluid instead of in the aspirated solution. The amount of protein pipetted thus varies very significantly between drops dispensed at the beginning and at the end of a 96-well plate. The protein concentration is therefore not constant within a plate and this may be one reason why some researchers have found it difficult to scale up to larger drops (see Brown *et al.*, 2003).

Most of the mixing probably occurs within the solenoid valve. When aspirating 15 µl, some of the aspirated solution will enter the valve and extensive mixing can then take place as the valve opens and closes. Preliminary experiments suggest that this mixing is reduced by aspirating smaller volumes (presumably retaining the entire sample within the tip). A small air gap can be introduced to separate the sample from the system fluid, but the air gap can be broken up within the valve and can therefore only be used with smaller aspirated volumes. In experiments with 50 nl drops and a 500 nl air gap, we find that the pipetting becomes very unreliable.

3.7. Drop evaporation

A major concern when using nanolitre droplets in crystallization experiments is the evaporation of the drops during the pipetting process before the plate can be sealed. With our protocol the Cartesian instrument pipettes the protein rapidly 'on-the-fly', but pipetting all the reservoir solutions is relatively slow. The humidity chamber available from the Cartesian supplier did not seem adequate for our application; instead a simple cover was designed (see §2.2.3 and Fig. 1) and all experiments are now carried out with this in place.

To evaluate if the anti-evaporation cover is effective, drop evaporation rates were monitored by measuring the drop diameter under a microscope. 50 and 100 nl drops of water and 30% aqueous 2-propanol were investigated for four different setups: drops on an open flat surface, drops on Greiner plates in the absence and presence of the corresponding reservoir solution, and drops on Greiner plates in the presence of reservoir solution and with the anti-evaporation cover in place. It can be seen from Fig. 8 that without the anti-evaporation cover most drops had completely evaporated within 12 min, the time required for the instrument to complete one plate. As expected, the 50 nl 2-propanol droplets evaporated fastest. Using the anti-evaporation cover virtually eliminated the evaporation problem and drop volumes remained constant.

The 30% 2-propanol droplets in the absence of the cover had a consistently smaller diameter at time = 0 than corresponding droplets with the cover in place (Figs. 8*c* and *d*). It is likely that most of the 2-propanol had already evaporated before the table had moved the plate to the inspection point under the microscope. The 30% 2-propanol droplets therefore seem to lose volume in two stages: firstly the 2-propanol

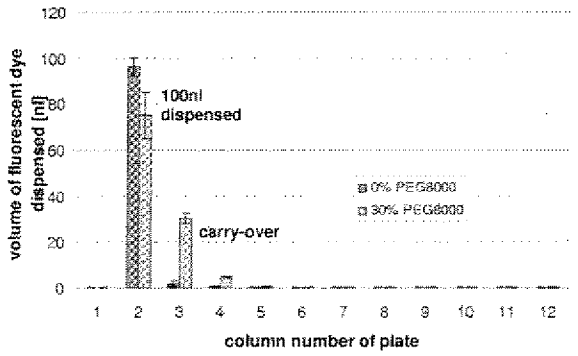

Figure 6
Carry over between wells when dispensing reservoir solutions of buffer alone and buffered 30% PEG8000. The fluorescent dye was added to reservoir solutions in column 2 and the volume of dye pipetted was measured for drops in all columns. Each point represents the average volume (and its standard deviation) of all eight tips. The standard protocol for dispensing the reservoir solutions was used. The buffer was 20 m*M* Tris-HCl, pH 8.0.

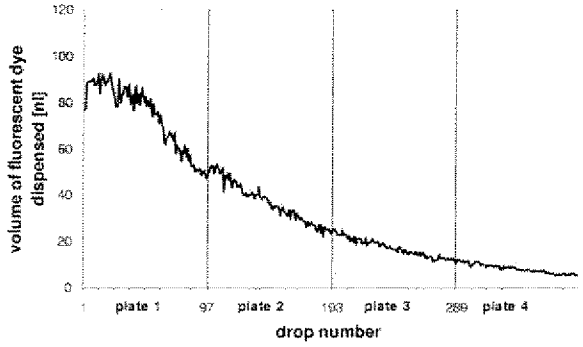

Figure 7
Mixing of sample with system fluid. The fluorescent dye was present in the aspirated sample but not in the system fluid (both solutions were buffered, see §2). The volume of dye pipetted was measured as function of drop number. The standard protocol for dispensing the protein solution was used: the aspirated volume was 15 µl and the drop volume 100 nl. Pipetting was carried out over four plates to record the fall-off in fluorescence intensity. Volumes shown are individual measurements as pipetted by tip No. 7. Small divisions mark rows of 12 drops.

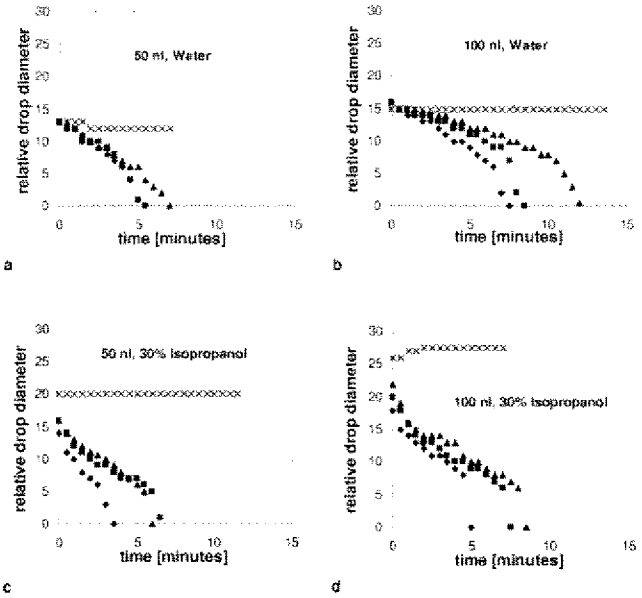

Figure 8
Evaporation of 50 and 100 nl drops of water, (*a*) and (*b*), and 30% 2-propanol, (*c*) and (*d*), when dispensed onto different supports: flat open surface (diamonds); Greiner plate, no solution in reservoir (squares); Greiner plate, with corresponding reservoir solution (triangles); Greiner plate, with corresponding reservoir and anti-evaporation cover (crosses).

evaporates rapidly within a few seconds and then the water evaporates more slowly, taking several minutes. The diameters of the 2-propanol drops were larger than the water drops due to the lower surface tension of 2-propanol.

4. Conclusions and future work

We have described a protocol that allows crystallization experiments to be set up at a throughput of up to about 4000 drops in a working day using commercially available equipment with only minor modifications. A very significant advantage of the present protocol over those previously used in our laboratory is the order of magnitude reduction in the volume of protein solution required to set up a given crystallization screen. There is still scope for improvement in several areas; for instance, there is no reason to believe that 100 nl is the optimum drop size. In addition there are still problems with the protocol, notably mixing of protein and system fluid during dispensing. Nevertheless, the current procedure has gained rapid and general acceptance among the local structural biology community, whose experiences are reported in the accompanying paper (Brown *et al.*, 2003).

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Like everything, for Pam.

is the major solute present, vapor diffusion (evaporation and condensation) in this closed system results in net transfer of water from the protein solution in the drop to the reservoir, until the precipitant concentration is the same in both solutions. Because the reservoir is much larger than the protein solution, the final concentration of the precipitant in the drop is nearly equal to that in the reservoir. When the system comes to equilibrium, net transfer of water ceases, and the protein solution is maintained at constant precipitant concentration. At this point, drop shrinkage has increased both [precipitant] and [protein], moving conditions diagonally into the nucleation region (blue circle in Fig. 3.5*b*). In this way, the precipitant concentration in the protein solution rises to the level required for nucleation and remains there without overshooting because, at equilibrium, the vapor pressure in the closed system equals the inherent vapor pressure of both protein solution and reservoir. As nuclei form, the protein concentration decreases, moving the conditions vertically into the growth region (green circle in Fig. 3.5*b*).

Frequently the crystallographer obtains many small crystals instead of a few that are large enough for diffraction measurements. If many crystals grow at once, the supply of dissolved protein will be depleted before crystals are large enough to be useful. Small crystals of good quality can be used as seeds to grow larger crystals. The experimental setup is the same as before, except that each hanging droplet is seeded with a few small crystals. Seed crystals are sometimes *etched* before use by brief soaking in buffer with precipitant concentration lower than that of the mother liquor. This soak dissolves outer layers of the seed crystal, exposing fresh surface on which crystallization can proceed. Seeds may also be obtained by crushing small crystals or by stroking a crystal with a hair and passing the hair through the crystallization droplet (it is reported that animal whiskers are best—really). Whatever the seeding method, crystals may grow from seeds up to ten times faster than they grow anew, so most of the dissolved protein goes into only a few crystals.

3.3.3 Growing derivative crystals

Crystallographers obtain the derivatives needed for phase determination and for studying protein-ligand interactions by two methods: cocrystallizing protein and ligand, and soaking preformed protein crystals in mother-liquor solutions containing ligand.

It is sometimes possible to obtain crystals of protein-ligand complexes by crystallizing protein and ligand together, a process called *cocrystallization*. For example, a number of NAD^+-dependent dehydrogenase enzymes readily crystallize as NAD^+ or NADH complexes from solutions containing these cofactors. Cocrystallization is the only method for producing crystals of proteins in complexes with large ligands, such as nucleic acids or other proteins.

A second means of obtaining crystals of protein-ligand complexes is to soak protein crystals in mother liquor that contains ligand. As mentioned earlier, proteins retain their binding and catalytic functions in the crystalline state, and ligands can diffuse to active sites and binding sites through channels of water in the crystal. Soaking is usually preferred over cocrystallization when the crystallographer plans

to compare the structure of a pure protein with that of a protein-ligand complex. Soaking preformed protein crystals with ligands is more likely to produce crystals of the same form and unit-cell dimensions as those of pure protein, so this method is recommended for first attempts to make isomorphic heavy-atom derivatives.

Making selenomet derivatives requires taking advantage of modern methods of molecular biology, in which the gene encoding a desired protein is introduced (for example, on a plasmid) into a specially designed strain of bacterium or other microbe, which is called an *expression vector*. The microbe, in turn, *expresses* the gene, which means that it produces messenger RNA from the gene and synthesizes the desired protein. To produce a selenomet derivative, the gene for the desired protein is expressed in a mutant microbe that cannot make its own methionine, and thus can live only in a growth medium that provides methionine. If the growth medium provides selenomethionine instead of methionine, the microbe usually grows normally, and expression results in incorporation of selenomethionine wherever methionine would normally appear. Purification and crystallization of the selenomet derivative usually follow the same procedures as for the native protein.

Finally, some proteins naturally contain metal ions that can serve the same purpose in phasing as introduced heavy-atom compounds. For example, hemoglobin contains iron (II) ions that can be used to obtain phase information. For such proteins, there is often no need to produce heavy-atom or selenomet derivatives.

3.3.4 Finding optimal conditions for crystal growth

The two most important keys to success of a crystallographic project are purity and quantity of the macromolecule under study. Impure samples will not make suitable crystals, and even for proteins of the highest purity, repeated trials will be necessary before good crystals result.

Many variables influence the formation of macromolecular crystals. These include obvious ones like protein purity, concentrations of protein and precipitant, pH, and temperature, as well as more subtle ones like cleanliness, vibration and sound, convection, source and age of the protein, and the presence of ligands. Clearly, the problem of developing a reliable source of crystals entails controlling and testing a large number of parameters. The difficulty and importance of obtaining good crystals has prompted the invention of crystallization robots that can be programmed to set up many trials under systematically varied conditions.

The complexity of this problem is illustrated in Fig. 3.7, which shows the effects of varying just two parameters, the concentrations of protein (in this case, the enzyme lysozyme) and precipitant (NaCl). Notice the effect of slight changes in concentration of either protein or precipitant on the rate of crystallization, as well as the size and quality of the resulting crystals.

A sample scheme for finding optimum crystallization conditions is to determine the effect of pH on precipitation with a given precipitant, repeat this determination at various temperatures, and then repeat these experiments with different precipitating agents. Notice in Fig. 3.7 that the region of [protein] versus [precipitant] that gives best crystals is in the shape of an arc, like the arc-shaped growth region

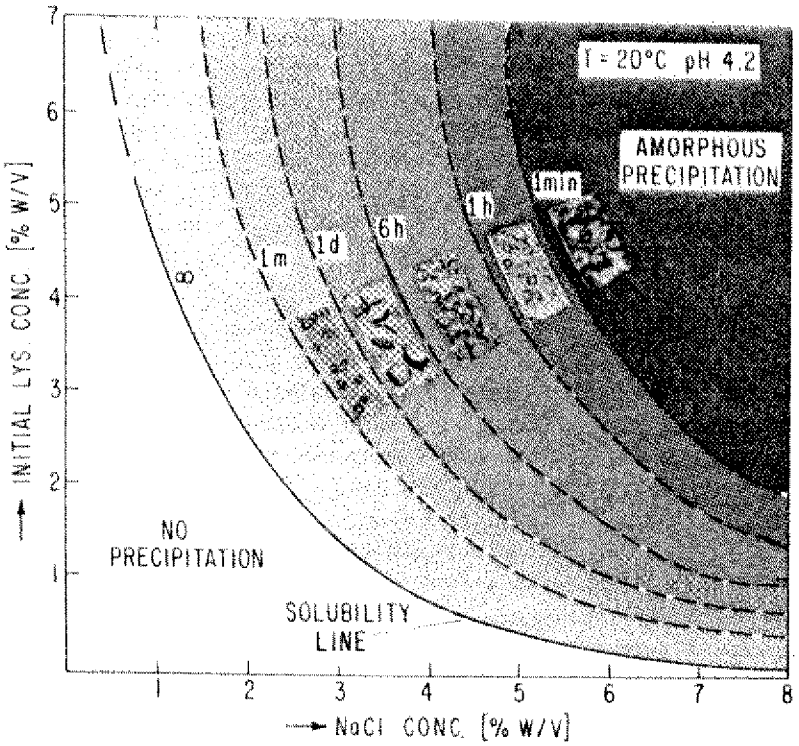

Figure 3.7 ▶ Schematic map of crystallization kinetics as a function of lysozyme and NaCl concentration obtained from a matrix of dishes. Inserts show photographs of dishes obtained one month after preparation of solutions. From G. Feher and X. Kam, in *Methods in Enzymology* **114**, H. W. Wyckoff, C. H. W. Hirs, and S. N. Timasheff, eds., Academic Press, Orlando, Florida, 1985, p. 90. Photo and caption reprinted with permission.

of Fig. 3.5*a*. It turns out that if these same data are plotted as [protein] versus ([protein] × [precipitant]), this arc-shaped region becomes a rectangle, which makes it easier to survey the region systematically. For such surveys of crystallization conditions, multiple batches of crystals can be grown conveniently by the hanging-drop or other methods in crystallization plates of 24, 48, or 96 wells (Fig. 3.8), each with its own cover. This apparatus has the advantage that the growing crystals can be observed through the cover slips with a dissecting microscope. Then, once the ideal conditions are found, many small batches of crystals can be grown at once, and each batch can be harvested without disturbing the others.

Crystallographers have developed sophisticated schemes for finding and optimizing conditions for crystal growth. One approach, called a response-surface procedure, begins with the establishment of a scoring scheme for results, such as giving higher scores for lower ratios of the shortest to the longest crystal dimension. This method gives low scores for needles and higher scores for cubes.

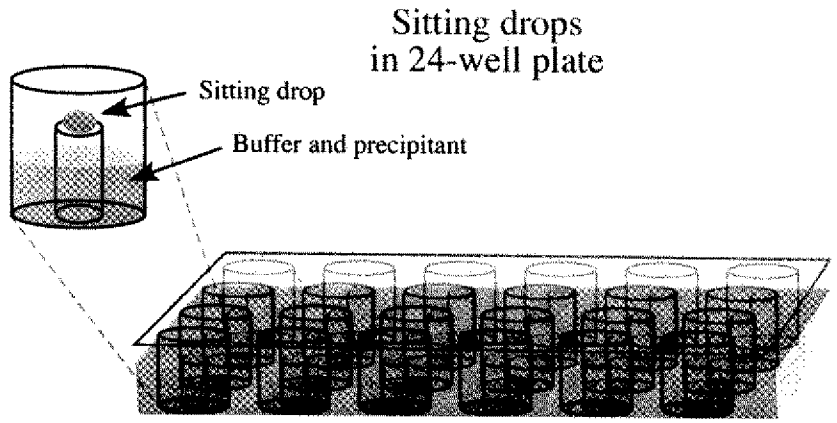

Figure 3.8 ▶ Well-plate, in which 24 sitting-drop crystallization trials can be carried out. Each well contains a pedestal with a concave top, in which the drop sits. Vapor diffusion occurs between drop and reservoir in the bottom of the well.

Then crystallization trials are carried out, varying several parameters, including pH, temperature, and concentrations of protein, precipitant, and other additives. The results are scored, and the relationships between parameters and scores are analyzed. These relationships are fitted to mathematical functions (like polynomials), which describe a complicated multidimensional surface (one dimension for each variable or for certain revealing combinations of variables) over which the score varies. The crystallographer wants to know the location of the "peaks" on this surface, where scores are highest. Such peaks may lie at sets of crystallization conditions that were not tried in the trials and may suggest new and more effective conditions for obtaining crystals. Finding peaks on such surfaces is just like finding the maximum or minimum in any mathematical function. You take the derivative of the function, set it equal to zero, and solve for the values of the parameters. The sets of values obtained correspond to conditions that lie at the top of mountains on the surface of crystal scores.

An example of this approach is illustrated in Fig. 3.9. The graph in the center is a two-dimensional slice of a four-dimensional surface over which [protein], ([protein] × [precipitant]), pH, and temperature were varied, in attempts to find optimal crystallization conditions for the enzyme tryptophanyl-tRNA synthetase. Note that this surface samples the rectangular region [protein] versus ([protein] × [precipitant]), mentioned earlier. The height of the surface is the score for the crystallization. Surrounding the graph are photos of typical crystals obtained in multiple trials of each set of conditions. None of the trial conditions were near the peak of the surface. The photos labeled Opt1 and Opt2 are of crystals obtained from conditions defined by the surface peak. In this instance, the response-surface approach predicted conditions that produced better crystals than any from the trials that pointed to these conditions.

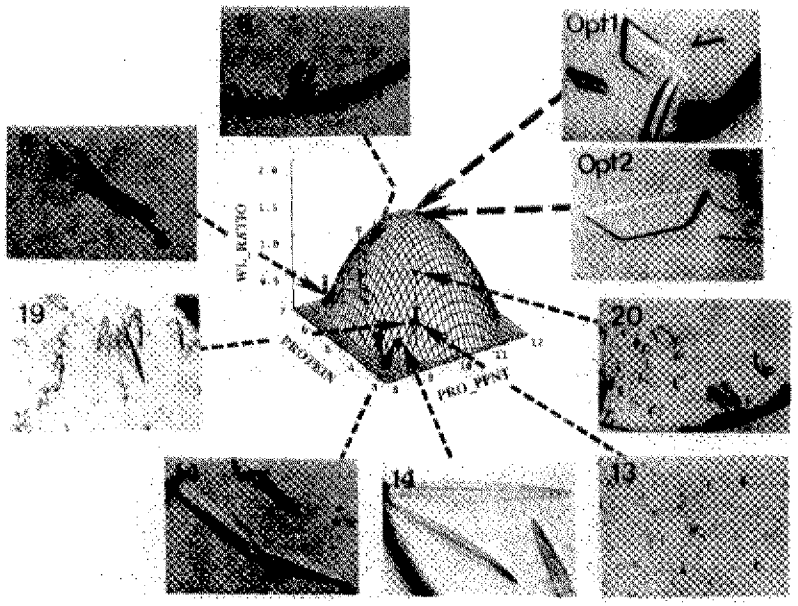

Figure 3.9 ▶ Optimization of conditions for crystallization of tryptophanyl-tRNA synthetase. Photo insets show crystals obtained from various conditions represented by points on the surface. Coordinates of the surface are protein concentration (PROTEIN), product of protein concentration and precipitant concentration (PRO_PPNT), and the shape of the crystal as reflected by the ratio of its two smallest dimensions, width and length (WL_RATIO). From C. W. Carter, in *Methods in Enzymology* **276**, C. W. Carter and R. M. Sweet, eds., Academic Press, New York, 1997, p. 75. Reprinted with permission.

So if you decide to try to grow some of your own crystals, how should you proceed? Theoretical studies like those described above, as well as the recorded experience of myriad crystallization successes and failures, have led to development of commercial screening kits that can often streamline the pursuit of crystals. Typical kits are sets of 24, 48, or 96 solutions containing various buffers, salts, and precipitants, representing a wide variety of potential crystallization conditions. After establishing appropriate protein concentration for screening (there is a kit for that, too), you would set up one trial with each of the screen solutions in cells of crystallization plates like the one shown in Fig. 3.8 ($ome kit$ even come with prefilled well plate$). If a particular screen solution produces promising crystals, you can then try to optimize the conditions by varying pH, [salt] or [precipitant] around the values of the screen solution.

Another way to tap accumulated wisdom about crystallization is through online databases. For example, at the combined Biological Macromolecule Crystallization Database and NASA Archive for Protein Crystal Growth Data (see CMCC home page), you can search for successful crystallization conditions for thousands

of macromolecules. You can search by many criteria, including molecule name, source species, prosthetic groups, molecular weight, space groups, as well as specific precipitants, methods, or conditions. Conditions that have succeeded with proteins similar to your target may be good starting points.

When varying the more conventional parameters fails to produce good crystals, the crystallographer may take more drastic measures. Sometimes limited digestion of the protein by a proteolytic enzyme removes a disordered surface loop, resulting in a more rigid, hydrophilic, or compact molecule that forms better crystals. A related measure is adding a ligand, such as a cofactor, that is known to bind tightly to the protein. The protein-ligand complex may be more likely to crystallize than the free protein, either because the complex is more rigid than the free protein or because the cofactor induces a conformational change that makes the protein more amenable to crystallizing. Desperation has even prompted addition of coffee (usually readily at hand in research labs) to precipitant mixtures, but I am aware of no successes from this measure.

Many membrane-associated proteins will not dissolve in aqueous buffers and tend to form amorphous precipitates instead of crystals. The intractability of such proteins often results from hydrophobic domains or surface regions that are normally associated with the interior of membranes. Such proteins have sometimes been crystallized in the presence of detergents, which coat the hydrophobic portion and decorate it with ionic groups, thus rendering it more soluble in water. A small number of proteins have been diffused into crystalline phases of lipid to produce ordered arrays that diffracted well and yielded structures. In some cases, limited proteolysis of membrane-associated proteins has removed exposed hydrophobic portions, leaving crystallizable fragments that are more like a typical water-soluble protein. Membrane proteins are greatly under-represented in the Protein Data Bank, due to their resistance to crystallization. The search for widely applicable conditions for crystallizing membrane proteins is one of crystallography's holy grails. The announcement of a model of a new membrane protein is usually greeted with much attention, and the first question is usually, "How did they crystallize it?"

The effects of modifications of the target protein, as well as the potential crystallizability of a newly purified protein, can be tentatively assessed before crystallization trials begin, through analysis of laser light scattering by solutions of the macromolecule. Simple, rapid light-scattering experiments (see Sec. 9.3, p. 219) can reveal much about the nature of the substance in solutions of varied composition, pH, and temperature, including estimates of average molecular mass of the particles, radius of gyration (dependent on shape of particles), rates of diffusion through the solution, and range and distribution of particle sizes (degree of *polydispersity*). Some of the measured properties correlate well with crystallizability. In particular, *monodisperse* preparations—those containing particles of uniform size—are more promising candidates for crystallization than those in which the protein is polydisperse. In many cases, polydispersity arises from nonspecific interactions among the particles, which at higher concentrations is likely to result in random aggregation rather than orderly crystallization

When drastic measures like proteolysis are required to yield good crystals, the crystallographer is faced with the question of whether the resulting fragment is worthy of the arduous effort to determine its structure. This question is similar to the basic issue of whether a protein has the same structure in crystal and in solution, and the question must be answered in the same way. Specifically, it may be possible to demonstrate that the fragment maintains at least part of the biological function of the intact molecule, and further, that this function is retained after crystallization.

3.4 Judging crystal quality

The acid test of a crystal's suitability for structure determination is, of course, its capacity to give sharp diffraction patterns with clear reflections at large angles from the X-ray beam. Using equipment typical of today's crystallography laboratories, researchers can collect preliminary diffraction data quickly and decide whether to obtain a full data set. However, a brief inspection of crystals under a low-power light microscope can also provide some insight into quality and can help the crystallographer pick out the most promising crystals.

Desirable visible characteristics of crystals include optical clarity, smooth faces, and sharp edges. Broken or twinned crystals sometimes exhibit dark cleavage planes within an otherwise clear interior. Depending on the lattice type (Chapter 4) and the direction of viewing relative to unit-cell axes, some crystals strongly rotate plane-polarized light. This property is easily observed by examining the crystal between two polarizers, one fixed and one rotatable, under a microscope. Upon rotation of the movable polarizer, a good-quality crystal will usually brighten and darken sharply.

Once the crystallographer has a reliable source of suitable crystals, data collection can begin.

3.5 Mounting crystals for data collection

The classical method of mounting crystals is to transfer them into a fine glass capillary along with a droplet of the mother liquor. The capillary is then sealed at both ends and mounted onto a goniometer head (see Fig. 4.25, p. 81, and Sec. 4.3.4, p. 80), a device that allows control of the crystal's orientation in the X-ray beam. The droplet of mother liquor keeps the crystal hydrated.

For many years, crystallographers have been aware of the advantages of collecting X-ray data on crystals at very low temperatures, such as that of liquid nitrogen (boiling point −196°C). In theory, lowering the temperature should increase molecular order in the crystal and improve diffraction. In practice, however, early

attempts to freeze crystals resulted in damage due to formation of ice crystals. Then crystallographers developed techniques for flash freezing crystals in the presence of agents like glycerol, which prevent ice from forming. Crystallography at low temperatures is called *cryocrystallography* and the ice-preventing agents are called *cryoprotectants*. Other cryoprotectants include xylitol or sugars such as glucose. Some precipitants, for example, polyethylene glycol, also act as cryoprotectants, and often it is only necessary to increase their concentration in order to achieve protection from ice formation.

If the crystal was not grown in cryoprotectant, preparation for cryocrystallography typically entails placing it in a cryoprotected mother liquor for 5–15 seconds to wash off the old mother liquor (this liquid is sometimes called a *harvest buffer*). If sudden exposure to cryoprotectant damages the crystal, it might be serially transferred through several solutions of gradually increasing cryoprotectant concentration. After transfer into protectant, the crystal is picked up in a small (< 1 mm) circular loop of glass wool or synthetic fiber, where it remains suspended in a thin film of solvent, sort of like the soap film in a plastic loop for blowing soap bubbles. The crystal is then flash frozen by dipping the loop into liquid nitrogen. If flash-freezing is successful, the liquid film in the loop freezes into a glass and remains clear (if it is frosty, crystalline water has formed, usually destroying the crystal in the process). For data collection, the loop is mounted onto the goniometer (see Fig. 4.25*b*, p. 81), where it is held in a stream of cold nitrogen gas coming from a reservoir of liquid nitrogen. A temperature of −100°C can be maintained in this manner.

In addition to better diffraction, other benefits of cryocrystallography include reduction of radiation damage to the crystal and hence the possibility of collecting more data—perhaps an entire data set—from a single crystal; reduction of X-ray scattering from water (resulting in cleaner backgrounds in diffraction patterns) because the amount of water surrounding the crystal is far less than that in a droplet of mother liquor in a capillary; and the possibility of safe storage, transport, and reuse of crystals. Crystallographers can take or ship loop-mounted flash-frozen crystals, in liquid-nitrogen-filled insulated containers, to sites of data collection, minimizing handling of crystals at the collection site. With all these benefits, it is not surprising that cryocrystallography is now common practice.